



November 7, 2018

PROTOCOL #1809402-404

**GLP VIRUCIDAL EFFICACY EVALUATION OF TWO HARD SURFACE DISINFECTANT
GERMICIDAL SPRAY PRODUCTS**

Prepared for:

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- 1.0 **TITLE:** GLP VIRUCIDAL EFFICACY EVALUATION OF TWO HARD SURFACE DISINFECTANT GERMICIDAL SPRAY PRODUCTS
- 2.0 **SPONSOR:** VIROX TECHNOLOGIES (SPONSOR)
2770 Coventry Road
Oakville, Ontario L6H 6R1
Canada
- 3.0 **TESTING FACILITY:** BIOSCIENCE LABORATORIES, INC.
1755 South 19th Avenue
Bozeman, Montana 59718
- 4.0 **STUDY DIRECTOR:** Volha Teagle, Ph.D.
- 5.0 **PROPOSED EXPERIMENTAL START DATE:** November, 2018
- 6.0 **PROPOSED EXPERIMENTAL COMPLETION DATE:** December, 2018
- 7.0 **PURPOSE:**

The purpose of this study is to evaluate virucidal efficacy of two batches of each of two test formulations when challenged with Canine Influenza A H3N2 and Feline Leukemia virus. Testing will be based upon methods described in as specified in the Canadian guidance document, Guidance document - Safety and efficacy requirements for hard surface disinfectant drugs (January, 2014), the American Society for Test Materials (ASTM) test method designated E1053-11, Standard Test Method to Assess Virucidal Activity of Chemicals Intended for Disinfection of Inanimate, Nonporous Environmental Surface, as specified in U.S. Environmental Protection Agency, Office of Chemical Safety and Pollution Prevention, OCSPP 810.2200, Disinfectants for Use on Hard Surfaces - Guidance for Efficacy Testing (February, 2018) and based upon the Official Methods of Analysis, 961.02, *AOAC Germicidal Spray Products as Disinfectants*. All testing will be performed in accordance with Good Laboratory Practices, as specified in 40 CFR 160, with the exception that the Study Sponsor retains responsibility for the determination of the identity, strength, purity, composition, and stability of the test formulation.

8.0 **SCOPE:**

This study will evaluate the virucidal efficacy of two batches of a ready-to-use spray disinfectant formulation and two batches of a concentrated spray disinfectant formulation, when used on dry, non-porous, inanimate surfaces. The concentrated spray disinfectant formulation will be diluted with sterile tap water. The test formulations will be evaluated Canine Influenza A H3N2 (#004-IDV, National Veterinary Services Laboratories, USDA) and Feline Leukemia virus strain CT1600 (ATCC #VR-1373) in the presence of an Organic Soil Load (OSL). A challenge suspension will be used to inoculate the bottom part of 100 mm X 15 mm glass Petri Dish carriers (one carrier per batch) to yield a minimum of 10^4 viruses per carrier following drying. After drying, each carrier will be treated using a spray application from a distance of 6 to 8 inches until thoroughly wet (3-4 sprays) and exposed at room temperature for the specified for each test formulation exposure time. Following the timed exposure, the neutralizer appropriate for test formulation will be added to the carrier. An aliquot of the neutralized suspension will be serially diluted in medium and assayed for the presence of viable viruses using the susceptible to the virus cell culture. The viral titers will be determined using a 50% tissue culture infectious dose (TCID₅₀) calculation -- the Quantal test (Spearman-Kärber Method).

9.0 TEST MATERIALS:

The test product to be evaluated will be provided to the Testing Facility by the Study Sponsor, complete with appropriate documentation. If the product name and/or lot number are not documented below, the information will be presented in the Final Report. Responsibility for the determination of the identity, strength, purity, composition, stability, and solubility of the test product, as well as the retention of the test product, rests with the Sponsor.

Test Product #1: Oxy-1 RTU
Active Ingredients: Hydrogen Peroxide

Batch Number: 1
Lot Number: 13657
Manufacture Date: 10/16/2018
Expiration Date: 10/16/2019

Batch Number: 2
Lot Number: 13658
Manufacture Date: 10/16/2018
Expiration Date: 10/16/2019

Test Product #2: Accel (Concentrate)
Active Ingredients: Hydrogen Peroxide

Batch Number: 1
Lot Number: 13659
Manufacture Date: 10/17/2018
Expiration Date: 10/17/2019

Batch Number: 2
Lot Number: 13660
Manufacture Date: 10/17/2018
Expiration Date: 10/17/2019

10.0 TEST CONDITIONS:

- | | | |
|------|---------------------------------------|--------------|
| 10.1 | Exposure Time/Test Product: Oxy-1 RTU | 1 minute |
| | Exposure Time/Test Product: Accel | 5 minutes |
| 10.2 | Exposure Temperature: | 22 °C ± 2 °C |

11.0 EQUIPMENT:

- 11.1 CO₂ Incubator, Temperature Range 37 °C ± 2 °C
- 11.2 Incubator Thermometers
- 11.3 Digital titrator (HACH)
- 11.4 Portable Pipettor
- 11.5 Continuously Adjustable Pipettes, 100 µL – 1000 µL Capacity
- 11.6 Continuously Adjustable Pipettes, 20 µL – 200 µL Capacity
- 11.7 Inverted Compound Microscope
- 11.8 Fluorescent Inverted Microscope
- 11.9 Laminar Flow Biological Safety Cabinet
- 11.10 Waste Pan
- 11.11 Calibrated Minute/Second Timers

12.0 SUPPLIES:

- 12.1 Sterile Disposable Pipettes
- 12.2 Sterile Polystyrene Test Tubes
- 12.3 Sterile Universal 1.0 mL and 0.2 mL Pipette Tips
- 12.4 Sterile, Powder-Free Gloves
- 12.5 Sterile Tissue Culture Treated 24-Well Plates
- 12.6 Sterile Tissue Culture Treated Glass Bottom 24-Well Plates
- 12.7 Viral Suspension
- 12.8 Sterile 100 μ L and 1000 μ L Displacement Tips
- 12.9 Sterile Flasks
- 12.10 Sterile 50 mL Centrifuge Tubes
- 12.11 Sterile Pipette Reservoir
- 12.12 Non-Sterile Waste Beaker for discarded tips, etc.
- 12.13 Sterile Cell Scrapers
- 12.14 Sterile Glass Petri Dishes

13.0 MEDIA:

- 13.1 1X Minimum Essential Medium (MEM)
- 13.2 Trypsin/EDTA
- 13.3 Phosphate Buffered Saline (PBS)
- 13.4 Antibiotics
- 13.5 Product Neutralizer
- 13.6 Horse Serum
- 13.7 Fetal Bovine Serum (FBS)
- 13.8 TPCK treated Trypsin

14.0 ORGANIC SOIL LOAD:

Fetal Bovine Serum (FBS), at the final concentration of $\geq 5\%$.

15.0 DILUENT:

200ppm sterile tap water will be used to dilute the Test Product #2. Prior to use, tap water hardness will be titrated using digital titrator and adjusted to at least 200ppm.

16.0 CHALLENGE VIRAL STRAINS:

- 16.1 Canine Influenza A H3N2 (NVSL USDA # 004-IDV)
 - 16.2 Feline Leukemia virus strain CT1600 (ATCC #VR-1373)
- NVSL = National Veterinary Services Laboratories
ATCC = American Type Culture Collection

17.0 HOST CELLS:

- 17.1 MDCK (Madin Darby Canine kidney cells, ATCC # CCL-34)
- 17.2 CRFK (Feline kidney cells, ATCC #CCL-94).

18.0 HOST CELL PREPARATION:

Cell lines will be maintained as monolayers in disposable cell culture labware and will be used for testing of Feline Calicivirus. Cell monolayers will be approximately 80% confluent and less than 48 hours old before inoculation with virus. Growth Medium (GM) and Maintenance Medium (MM) will be 1X MEM with appropriate supplements. Prior to plating, the GM will be replaced by MM.

19.0 TEST VIRUS PREPARATION:

Canine Influenza A H3N2 from BSLI high-titer virus stock and Feline Leukemia virus from ATCC stock will be used for this study. On the day of use, aliquots of the stock virus will be removed from a -70°C freezer and thawed. Prior to use in testing, FBS will be added to the test virus suspension to achieve a final concentration of $\geq 5\%$.

20.0 TEST PRODUCT PREPARATION:

On the day of testing, the Test Product #2 will be diluted as follows: 1 part of test product to 64 parts of sterile tap water. Prepared batches of formulation will be transferred into the provided spray bottles.

21.0 TEST PROCEDURE:

21.1 Preparation of Carriers

Sterilized glass Petri plates (100 mm x 15 mm) will be used as the carriers for this evaluation.

21.2 Contamination of Carriers

21.2.1 A 0.2 mL aliquot of the prepared virus suspension will be transferred to the bottom inside surface of a sterilized 100 mm x 15 mm glass Petri plate carrier. A sterile cell scraper will be used to spread the inoculum uniformly.

21.2.2 The virus suspension will be air-dried at room temperature until visibly dry.

21.2.3 One carrier per batch of the test formulation will be used.

21.3 Spray Formulation Test

21.3.1 After the inoculated carrier has dried, carriers will be treated with each batch of the test formulations using provided spray bottles. A spray apparatus will be adjusted so that the distance between the horizontal countertop and the product nozzle is 6 to 8 inches. The test formulation will be applied to each carrier by spraying three to four times from a distance of 6 to 8 inches. Sufficient test formulation will be applied to ensure that the carrier is thoroughly wetted. The carrier will be exposed to the test formulation at ambient temperature for the specified for each test product exposure, timed using a calibrated minute/second timer. Timing will commence after the spray procedure is completed. The treated carrier will be kept undisturbed for the duration of the contact time.

21.3.2 After the exposure time has elapsed, the appropriate amount of the neutralizer (not more than 20 mL) will be added to the Petri dish and the virus test formulation mixture will be scraped from the surface of the carrier using a sterile cell scraper. Subsequent 10-fold dilutions will be made in MM and plated in four replicates.

21.3.3 *Virus control.* Two carriers will be used for Virus Control. The test virus will be dried as described in Section 21.2.1 and 21.2.1. A total of 2.0 mL of MM will be added to the contaminated carriers. The carriers will be exposed to MM at ambient temperature for 1 and 5 minutes, timed using a calibrated minute/second timer. The appropriate neutralizer will be added to the carriers and the virus will be scraped from the surface. Subsequent 10-fold dilutions will be made in MM and plated in four replicates.

21.3.4 *Initial Population.* The test virus will be diluted in the Neutralizer, and subsequent 10-fold dilutions will be performed in MM. Each dilution will be plated in four replicates.

21.3.5 *Neutralization and Cytotoxicity Controls.* A 0.2 mL aliquot of medium will be transferred to the bottom inside surface of a sterilized 100 mm x 15 mm glass Petri plate carrier. A sterile cell scraper will be used to spread the medium uniformly. The medium will be air-dried at

room temperature until visibly dry. After drying, the carrier will be treated as described in Section 21.3.1. After the exposure time has elapsed, the appropriate neutralizer will be dispensed into the carriers. The neutralized liquid will be used for the Neutralization control (virus added) and Cytotoxicity Control (no virus added). The Neutralization Control will receive an aliquot of the test virus, followed by exposure for at least 1 minute (Test Product, Oxy-1 RTU) and at least 5 minutes (Test Product, Accel), subsequent 10-fold dilutions in MM, and plating in four replicates. The Cytotoxicity Control will receive no virus, will be diluted (10-fold) in MM, and plated in four replicates.

21.3.6 Cell Culture Control. Intact cell culture monolayers will serve as the control of cell culture viability. The Growth Medium will be replaced by MM in all cell culture control wells (minimum four wells).

21.3.7 The plates will be incubated for 5 to 22 days at $37^{\circ}\text{C} \pm 2^{\circ}\text{C}$ in a CO_2 incubator.

21.3.8 For Canine Influenza A H3N2, Cytopathic/cytotoxic effect will be monitored using an inverted compound microscope.

21.3.9 For Feline Leukemia Virus, IFA (immunofluorescent assay) will be used to determine the presence of the virus infected cells. Following incubation with the test samples, the wells will be washed with PBS and approximately 1.0 mL ice-cold acetone will be added per well for fixation. Following fixation the wells will be washed with PBS. The antibodies against Feline Leukemia Virus will be applied onto fixed cells and exposed for 1-hour (± 15 minutes) at room temperature. Plates will be washed with PBS at least twice. In case an excessive non-specific fluorescence is observed, the counter stain such as Trypan blue or other can be applied. The fluorescence will be rated using inverted fluorescent microscope.

22.0 CALCULATIONS:

22.1 Viral titers will be expressed as $-\text{Log}_{10}$ of the 50% titration end point for infectivity. To calculate the viral titer, a 50% tissue culture infectious dose (TCID_{50}) calculation of a 50% fluorescent focus forming unit dose (FFFD_{50}) - the Quantal test (Spearman-Kärber Method) - will be applied.

$$\text{Log TCID}_{50} \text{ (or FFFUD}_{50}) = L - d(s - 0.5)$$

Where:

L = $-\text{Log}_{10}$ of the lowest dilution;

d = difference between dilution steps;

s = sum of proportions of positive wells.

22.2 The Log_{10} and percent (%) of infectivity reductions will be calculated as follows:

$$\% \text{ Reduction} = \left[1 - \frac{\text{TCID}_{50} \text{ test}}{\text{TCID}_{50} \text{ virus control}} \right] \times 100$$

$$\text{Log}_{10} \text{ Reduction} = (\text{Log}_{10} \text{TCID}_{50} \text{ of the Virus Control}) - (\text{Log}_{10} \text{TCID}_{50} \text{ of the Virucidal Test})$$

23.0 TEST ACCEPTANCE CRITERIA:

A valid test requires: 1) at least $4.8 \log_{10}$ of TCID_{50} be recovered from the Virus control; 2) cells in the Cell Control wells be viable and attached to the bottom of the well; 3) the medium be free of contamination in all wells of the plate; 4) when cytotoxicity is evident, at least a $3 \log_{10}$ reduction in titer be demonstrated beyond the cytotoxic level; 5) the test formulation be fully neutralized, so the difference between the test virus titer in Initial Population and Neutralization Control does not exceed $1.0 \log_{10}$.

24.0 STATISTICAL ANALYSIS:

A statistical analysis will not be performed on the data derived from this evaluation.

25.0 FINAL REPORT:

A Final Report will be prepared by BioScience Laboratories, Inc., describing the results of the study in a clear and concise manner. The final report will include all items required by 40 CFR Part 160.185.

26.0 EXCEPTIONAL CONDITIONS:

The Sponsor will be notified by telephone, email, and/or letter of any exceptions encountered in this study. The exceptional conditions or occurrences will be detailed in full and formally recorded. Exceptional conditions that occur and are not addressed in this Protocol will be subject to Out-of-Scope charges (See Proposal/Contract).

27.0 LIABILITY AND INDEMNIFICATION:

The Testing Facility's liability to the Study Sponsor under this Protocol shall be limited to the price of this evaluation. The Study Sponsor shall be responsible to Study Participants (when applicable) and to other third parties for the fitness of the product for use as defined in the Study Protocol.

28.0 REFERENCES:

- 28.1 Guidance document - Safety and efficacy requirements for hard surface disinfectant drugs (January, 2014).
- 28.2 ASTM E1053-11, Standard Test Method to Assess Virucidal Activity of Chemicals Intended for Disinfection of Inanimate, Nonporous Environmental Surfaces.
- 28.3 U.S. Environmental Protection Agency, Office of Chemical Safety and Pollution Prevention, OCSPP, 810.2200: Disinfectants for Use on Hard Surfaces-Guidance for Efficacy Testing (February, 2018).
- 28.4 Official Methods of Analysis of AOAC International, Official Method 961.02, *AOAC Germicidal Spray Products as Disinfectants*.

29.0 DOCUMENTATION AND RECORD-KEEPING:

All documentation and records will be compiled, analyzed, and retained by BioScience Laboratories, Inc., at its facility in Bozeman, Montana. All raw data for this study will be retained in safe storage for the life of the product registration with the EPA, or as specified by the Sponsor.

30.0 PRODUCT DISPOSITION:

It is the responsibility of the Sponsor to retain a sample of the test substance(s) for future audit or evaluation. All unused test material will be disposed of following study completion, unless otherwise indicated by the Sponsor prior to initiation of the study.

31.0 QUALITY ASSURANCE AUDITS:

The Quality Assurance Unit (QAU) will conduct in-phase audits of critical processes in testing at least once and advise the Study Director and Management of the outcome. On completion of testing, the QAU will perform an audit of the data and of the Final Report in its entirety.

32.0 ACCEPTANCE:

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GERMICIDAL SPRAY PRODUCTS

ACCEPTED BY: BIOSCIENCE LABORATORIES, INC. (TESTING FACILITY)
1755 South 19th Avenue
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Study
Director:

Volha Teagle, Ph.D.

11-09-2018
Date of Study Initiation

ACCEPTED BY: VIROX TECHNOLOGIES (SPONSOR)
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11/9/18
Date

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